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BRCA1 is involved in repair of double strand breaks induced by ionizing radiation and chemotherapy drugs. BRCA1 and nuclear hormone receptors interact with p300 and CREB binding protein (CBP) to activate target gene transcription. Few studies have suggested a role for nuclear hormone receptors in DNA repair. E2 and RA had opposing effects on DNA damage and breast cancer cell survival following double strand break damage. Estradiol but not retinoic acid treatment resulted in complex formation between ERα, CBP, and BRCA1 in ER positive breast cancer cell lines. Mutant BRCA1 reduced DNA damage repair protein expression in human breast cancer cell lines. Mutant BRCA1 expression correlated with increased DNA damage and decreased repair activity in breast cancer cell lines but did not block nuclear hormone dependent effects. The truncated BRCA1 failed to form complexes with ERα and CBP which correlated with its ability to exert E2 independent effects on DNA repair. Mutant BRCA1 produced increased survival in breast cancer cells with DNA double strand breaks and inhibited cell cycle progression. Ectopic ERα expression was sufficient to produce the E2 mediated effects on DNA damage, repair, and survival.

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Table of Contents

INTRODUCTION

BRCA1, DNA Repair, and Breast Cancer. Breast cancer is one of the leading causes of death in women. The disease and its consequences are a significant cause of morbidity and mortality (Russo, 2000). Surgical removal of the tumor followed by radiotherapy is the therapeutic mainstay for early disease; however mastectomy with axillary lymph node dissection and chemotherapy is required for disseminated breast cancer. Inactivating mutations in the tumor suppressor BRCA1 have been discovered in familial forms of the disease and are associated with significantly increased risk of developing breast cancer (Yang and Lippman, 1999). The BRCA1 gene encodes a protein shows no significant similarity to previously described proteins with the exception of a RING zinc finger motif in the amino terminus and carboxyl terminal repeats (Bertwistle and Ashworth, 1998). The carboxyl terminal repeats are found in a range of proteins involved in DNA repair (Koonin et al., 1996; Callebaut and Mornon, 1997). BRCA1 has been shown to induce expression of the DNA damage response gene GADD45 (MacLachlan et al., 2000). Additionally, BRCA1 functionally associates with Rad51 protein which is involved in double strand break repair (Scully et al., 1997). This evidence suggests an important role for BRCA1 in DNA repair and maintaining genome integrity (Kinzler and Vogelstein, 1997; Brugarolas and Jacks, 1997). BRCA1 is involved in repair of double strand breaks induced by chemotherapy drugs (Husain et al., 1998). A number of chemotherapeutic agents used in the treatment of breast cancer produce their cytotoxic effects by creating DNA damage (Hoeijmakers, 2001).

Nuclear Hormone Receptors, Coactivators, and BRCA1. Among the most important nuclear hormone receptors expressed by breast cancer cells are those for estrogen and retinoic acid (Russo and Russo, 1998). Estrogens such as 17-β-estradiol (E2) have been shown to dramatically enhance proliferation of mammary gland epithelium (Huseby et al., 1984). In contrast, a number of natural and synthetic retinoids have been shown to inhibit proliferation of these cells and have been used as chemotherapy drugs in the treatment of breast cancer (Li et al., 1999). Estrogen receptors (ER) and retinoic acid receptors (RAR) are members of a family of ligand dependent transcription factors that include steroid, thyroid, and vitamin D receptors (Mangelsdorf et al., 1995). Both ER and RAR have functional domains for DNA binding, ligand binding, dimerization, and transcriptional activation. ER and RAR require coactivator proteins such as CREB binding protein (CBP) to activate target gene transcription. CBP interacts with ER and RAR in their ligand bound conformation to induce gene expression (Chakravarti et al., 1996). CBP has histone acetyltransferase activity, allowing for histone disassembly and transcriptional activation (Ogryzko et al., 1996). CBP has also been shown to interact with and enhance the function of BRCA1 (Pao et al., 2000).

A New Role for Estradiol and Retinoic Acid in BRCA1 Mediated DNA Repair. While the effects of E2 and RA on proliferation of human breast cancer cells have been known for many years, no studies have suggested a role for these hormones in DNA repair. The original application proposed a new mechanism by which ER and RAR regulate BRCA1 mediated DNA repair via CBP. This model may ultimately predict which breast cancers will respond to the inclusion of retinoids in the chemotherapy regimen.

BODY OF REPORT

In the third year of the funded application (April 2004-April 2005), we submitted a manuscript to a peer reviewed breast cancer journal which describes the results of the research funded by the first two years of this award. We have provided a detailed summary the final results below which correspond to the completion of Tasks 1 and 2 reported previously to this command. Reprints of the journal article with figures will be forwarded as soon as possible.

E2 and RA have been shown to have opposing effects on proliferation of human breast epithelium. However the effects of these nuclear hormones on DNA damage and repair have rarely been examined. We treated four human breast cancer cell lines with 100 nM E2 or RA followed by etoposide to induce double strand DNA breaks. Etoposide treatment resulted in 60-70% TUNEL positive cells within 16 hours. Pretreatment with E2 resulted in increased survival of ER positive MCF7 and T47D breast cancer cell lines (40% TUNEL positive cells) compared to vehicle treated control cultures. No effect of E2 was observed in ER negative MDA-MB-231 and MDA-MB-468 cell lines. In contrast, RA treatment increased the number of apoptotic cells to 80% in all cell lines. In cultures simultaneously treated with E2 and RA, the pro-survival effect of E2 was still observed in ER positive cells but not in ER negative lines. However no effects of E2 or RA were observed in cisplatin treated cultures indicating that the effects of these ligands were specific for survival following double strand breaks but not adduct formation. We concluded that E2 and RA had opposing effects on breast cancer cell survival following double strand DNA break damage.

To determine if the pro-survival effects of E2 were mediated by kinase signaling or second messengers, we treated ER positive MCF7 and T47D cells with selective inhibitors of these pathways prior to treatment with E2 and etoposide. Treatment with MEK, JNK, p38, Akt, PKC, PI3K, or PLC inhibitors had no effect on the pro-survival effect of E2 as determined by TUNEL assay. These results indicate that signaling pathways upstream of ER had no effect on the pro-survival effect of E2 in cells exposed to DNA double strand break damage.

To determine if the effects of E2 and RA on cell survival correlated with the extent of double strand break damage, we performed single cell gel electrophoresis on human breast cancer cell lines treated with these ligands prior to etoposide. E2 decreased the extent of DNA damage by 40% in ER positive cell lines. No effect of E2 on DNA damage was observed in ER negative cell lines. In contrast, RA increased relative DNA damage levels by 10-20% in all cell lines examined. In cells treated simultaneously with E2 and RA, relative DNA damage levels decreased similar to treatment with E2 alone. These results indicate that the cell survival effects of E2 and RA on human breast cancer cell lines correlate with relative DNA damage levels in cultures treated with these ligands followed by etoposide.

To determine if E2 and RA effects on DNA damage could result from changes in DNA repair activity, we analyzed plasmid end joining in ligand treated human breast cancer cell lines. E2 increased the number of transformants in the end joining assay by 20% using extract from ER positive cell lines. No effect of E2 was observed using extract from ER negative cell extract. RA treatment inhibited plasmid end joining in all

cell extracts by 30%. In extracts from cells treated simultaneously with E2 and RA, the number of transformants increased similar to that observed with E2 alone using extract from ER positive cells. These results indicate that the effects of E2 and RA on DNA damage correlated with DNA repair activity in human breast cancer cell lines.

To determine if the effects of E2 and RA on DNA repair activity were the result of changes in repair protein expression, we examined expression of double strand break repair gene products by western blot. E2 and RA did not affect expression of 7 double strand break repair proteins in ER positive and ER negative human breast cancer cell lines. These results indicated that the effects of E2 and RA on DNA repair activity were not the result of changes in repair protein expression. Therefore we wondered if ER and RAR coactivator proteins such as CBP may differentially associate with these receptors and regulators of DNA repair such as BRCA1 in human breast cancer cell lines. E2 treatment induced complex formation between ERα, BRCA1, and CBP in ER positive T47D cells. This complex was not observed in ER negative MDA-MB-468 cells treated with E2. RA treatment showed recruitment of CBP to RARα in both cell lines, but BRCA1 was not detected in these complexes. Low level association of BRCA1 with CBP was observed in vehicle treated cells, but neither ERα nor RARα were detected in these complexes. No protein interactions were observed when preimmune IgG was used in place of anti-CBP antibody in the immunoprecipitations. These results indicate that E2 treatment results in complex formation between ERα, CBP, and BRCA1 in ER positive breast cancer cell lines. RA treatment recruits CBP but not BRCA1 to RARα in both ER positive and negative cell lines.

Given that recruitment of BRCA1 to the ERα/CBP complex correlated with increased DNA repair and survival which was not observed in RA treated cells, we wanted to determine the contribution of BRCA1 to these processes. To accomplish this task, we stably transfected T47D and MDA-MB-468 breast cancer cells with a carboxyl terminal truncation mutant of BRCA1. This BRCA1 mutant lacked the BRCT repeat region believed to be involved in DNA repair. To determine the effects of the BRCA1 mutant on expression of double strand break repair proteins, we treated stable T47D and MDA-MB-468 mutant and control clones with etoposide for 16 hours. Etoposide treatment induced expression of Rad52, Rad54, XRCC2, XRCC3, and XRCC4 in T47D control clones. The mutant BRCA1 protein blocked induction of all five of these genes by etoposide. In contrast, expression of the mismatch repair protein MSH2 and the nucleotide excision repair gene product XPA was unaffected by the mutant BRCA1 or etoposide treatment. With the exception of XPA, these proteins were generally below the detection limit for western blot in MDA-MB-468 clones. These results indicate that BRCA1 induces DNA damage repair protein expression in human breast cancer cell lines with DNA double strand breaks.

To determine if the mutant BRCA1 protein could block the protective effects of E2 on ER positive breast cancer cell lines, we treated T47D stable clones with E2 or RA followed by etoposide. The ER negative MDA-MB-468 clones served as controls in these experiments. E2 and RA reproduced the effects on relative DNA damage levels in T47D control clones first seen in untransfected cells. In contrast, relative DNA damage levels were 2 fold higher in T47D clones expressing the mutant BRCA1 protein. However, the mutant BRCA1 was unable to block either the protective effects of E2 or the deleterious effects of RA on relative DNA damage levels in these cells. The E2 effect

again dominated in cultures treated simultaneously with E2 and RA. DNA damage was also greater in ER negative MDA-MB-468 clones expressing mutant BRCA1 but was unresponsive to E2. RA treatment increased relative DNA damage levels by 20% in these clones. These results indicate that mutant BRCA1 expression correlates with increased etoposide induced DNA damage in human breast cancer cell lines but does not block nuclear hormone dependent effects.

To determine if increased DNA damage as the result of mutant BRCA1 resulted from decreased repair activity, we used lysates from E2 and RA breast cancer clones in the end joining assay. Expression of the BRCA1 mutant decreased end joining by 60% using lysate from T47D clones. The mutant BRCA1 gene product did not block the effects of E2 and RA on end joining in this assay. Expression of the mutant BRCA1 also decreased end joining in MDA-MB-468 clones by 50%. RA treatment of these clones produced a 25% reduction in end joining in these assays, but E2 treatment had no effect in the ER negative clones. These results indicated that expression of the BRCA1 mutant resulted in decreased DNA repair activity in ER positive and ER negative breast cancer clones.

We expected the decreased DNA repair activity observed in BRCA1 mutant clones to correlate with decreased survival in breast cancer cells exposed to etoposide. Expression of the BRCA1 mutant resulted in increased survival of both T47D and MDA-MB-468 clones. Etoposide treatment produced only 35% TUNEL positive cells in T47D clones expressing the BRCA1 mutant construct compared to 50% in control cultures. Similarly, etoposide treatment resulted in 45% TUNEL positive MDA-MB-468 mutant cells compared to 60% of control clones. The pro-survival effects of E2 and pro-apoptotic effects of RA were not blocked by the BRCA1 mutant in T47D clones. RA also had pro-apoptotic effects on MDA-MB-468 clones expressing the BRCA1 mutant but E2 had no effect on the ER negative line. These results indicate that despite decreased DNA repair as the result of mutant BRCA1, this construct also produced increased survival in breast cancer cells with DNA double strand breaks.

BRCA1 has been shown to interact with CBP/p300 through its carboxyl terminus. We hypothesized that the failure of the mutant BRCA1 protein to affect E2 mediated DNA repair may be due to decreased ability of the truncated tumor suppressor to interact with CBP. To test this hypothesis we immunoprecipitated CBP from E2 and RA treated stable T47D and MDA-MB-468 clones expressing the truncated BRCA1 protein. The larger wild type BRCA1 protein immunoprecipitated with CBP in both T47D and MDA-MB-468 clones. However, the mutant BRCA1 protein was not detected in these immunoprecipitates even though it was detected in these cells when anti-BRCA1 antibody was used in the immunoprecipitation. ERα formed complexes with wild type BRCA1 and CBP in E2 treated T47D but not MDA-MB-468 clones, similar to that observed in the parental breast cancer cell lines. RARα associated with CBP but not wild type BRCA1 in RA treated T47D and MDA-MB-468 clones. These results indicate that the truncated BRCA1 fails to form complexes with ERα and CBP which correlates with its ability to exert E2 independent effects on DNA damage repair.

Overexpression of BRCA1 has been shown to induce growth arrest in dividing cells. Given that DNA damage agents target dividing cells, we hypothesized that cell cycle inhibition due to the mutant BRCA1 could result in greater resistance to etoposide. BrdU incorporation analysis demonstrated that the mutant BRCA1 transgene inhibited S

phase progression in both T47D (17% vs. 10% positive cells) and MDA-MB-468 (20% vs. 16%) lines. The effect of the BRCA1 mutant was greater than that of etoposide treatment of control clones. Etoposide treatment of BRCA1 clones further reduced BrdU incorporation (17% vs. 7% positive cells in T47D and 20% vs. 13% in MDA-MB-468 lines). We also examined expression of cell cycle regulatory proteins in both lines. Expression of the mutant BRCA1 reduced EGFR levels below the limit of detection for western blot in MDA-MB-468 clones. Simiarly, expression of the growth factor receptor c-met was completely inhibited in T47D clones expressing mutant BRCA1. Expression of the G2 phase protein cyclin B was reduced to undetectable levels in etoposide treated T47D clones expressing the mutant BRCA1 construct. Expression of the G1 phase protein cyclin E was inhibited greater than 95% in T47D clones expressing the mutant BRCA1. Etoposide treatment induced cdk2 levels in these clones which was inhibited 5 fold by the mutant BRCA1. This construct also reduced expression of the G1 kinases cdk4 and cdk6 to nearly undetectable levels in MDA-MB-468 clones. These results indicate that the mutant BRCA1 construct inhibited cell cycle progression which correlated with increased resistance to etoposide.

To determine if ERα was sufficient to confer E2 mediated DNA damage repair and increased survival to ER negative breast cancer cell lines, we stably transfected MDA-MB-468 cells with an ERα expression vector. Ectopic ERα formed complexes with BRCA1 and CBP in E2 treated MDA-MB-468 clones similar to that observed in T47D cells. RARα failed to form complexes with BRCA1 in RA treated cells. These clones were treated with E2 and RA alone or in combination prior to etoposide exposure. Ectopic ERα expression in MDA-MB-468 cells resulted in E2 mediated decreases in relative DNA damage levels of 25%. This effect was also observed when E2 and RA were used in combination. ERα expression in MDA-MB-468 clones had no effect on RA mediated DNA damage. G418 resistant MDA-MB-468 control clones did not exhibit E2 mediated decreases in relative DNA damage levels. The effects of E2 and RA in G418 resistant ER positive T47D clones were similar to that observed in the parental cell line. Decreased DNA damage correlated with increased DNA repair activity in E2 treated, ERα expressing MDA-MB-468 clones as demonstrated by the end joining assay. Results obtained using T47D and MDA-MB-468 G418 resistant control clones were similar to those observed in the parental cell lines. Increased etoposide resistance and survival was also observed in the E2 treated MDA-MB-468 clones. RA treatment decreased clonal survival similar to that observed in the MDA-MB-468 parental line. Results obtained using T47D and MDA-MB-468 control clones were similar to those observed in the parental cell lines. These results indicate that ectopic ERα expression was sufficient to produce the E2 mediated effects on relative DNA damage levels, DNA repair, and survival in etoposide treated MDA-MB-468 clones.

We also have made progress towards accomplishing Tasks 3a and 3b as outlined in the Statement of Work. We have induced mammary tumors in S-D rats using the MNU induction protocol detailed in the original application. The rats were treated with 100 mg/kg estradiol or retinoic acid to examine the effects of these ligands on DNA repair in the in vivo tumors (preparation for Task 3c). The induction protocol has resulted in 3-4 mammary tumors per animal which are primarily adenocarcinomas by histopathologic examination. Immunohistochemical analysis of ER, RAR, and BRCA1 expression in the

tumor tissue is ongoing. Task 3c (induction and evaluation of DNA double strand breaks in mammary tumor tissue) is currently in progress.

KEY RESEARCH ACCOMPLISHMENTS

We submitted a manuscript detailing the results of Tasks 1 and 2 to a peer reviewed breast cancer journal (see Reportable Outcomes).

Task 3a

The MNU protocol was used to induce mammary tumors in S-D rats as detailed in the original application. Animals were treated with estradiol or retinoic acid to determine the effects of these ligands on production of DNA double stranded breaks in mammary tumor tissue.

Task 3b

Histopathologic analysis of MNU induced rat mammary tumors revealed adenocarcinoma (3-4 tumors per animal on average). Immunohistochemical evaluation of ER, RAR, and BRCA1 expression is in progress.

Task 3c

In progress

REPORTABLE OUTCOMES

Crowe DL, Lee MK. New role for nuclear hormone receptors and coactivators in regulation of BRCA1 mediated DNA repair in breast cancer cell lines. Manuscript submitted

CONCLUSIONS

One of the key findings obtained through this award is the protective effects of E2 on ER positive breast cancer cell lines following DNA damage. This effect was ER dependent since stable transfection of this expression vector into ER negative breast cancer cell lines resulted in decreased DNA damage and increased survival when these cells were treated with E2 prior to etoposide. These results contrast with previous studies in which metabolites of E2 were shown to cause DNA damage by formation of direct adducts or generation of reactive oxygen species (Yager, 2000). Increased oxidative DNA damage has been detected in target tissues following estrogen exposure, and a low activity form of catechol-O-methyltransferase has been associated with increased risk of breast cancer. Glutathione depleted MCF7 cells treated with E2 exhibited significant increases in formation of 8-oxo-2'-deoxyguanosine (Mobley and Brueggemeier, 2002). Treatment of MCF7 cells with E2 resulted in decreased ability to metabolize peroxide and increased sensitivity to peroxide induced DNA damage (Mobley and Brueggemeier, 2004). These effects were not observed in ER negative breast cancer cell lines. Anti-estrogens have been shown to activate the detoxifying enzyme quinone reductase and protect against E2 mediated DNA damage (Bianco et al., 2003). Our present study does not rule out these DNA damage effects, but suggests a new role for E2 in DNA damage repair and cell survival which is regulated by complex formation with coactivator proteins and BRCA1.

Double strand DNA breaks have been shown to induce a number of growth factor signaling pathways (Reardon et al., 1999). However, we determined that the protective effects of E2 were not dependent on a number of upstream kinases and second messengers. It has been known for many years that ER is phosphorylated by MAPK (Arnold et al., 1995). Since then, ER has been shown to be a substrate for other kinases such as cdk2 and Akt which increase transcriptional activation of the receptor (Rogatsky et al., 1999). However, our data suggests that the actions of these kinases on ER transcriptional activation may not be required to protect breast cancer cell lines against DNA damage and E2 did not induce expression of double strand break repair genes. It will be interesting to determine if ER mutants lacking phosphorylation sites or transcriptional activation domains can inhibit the effects of E2 on double strand break repair and breast cancer cell survival.

BRCA1 is phosphorylated by ATM kinase which detects double strand DNA breaks (Valerie and Povirk, 2003). BRCA1 is phosphorylated at carboxyl terminal serine residues and colocalizes with histone H2AX and Rad proteins at sites of double strand break repair (Paull et al., 2000). BRCA1 null cells are sensitive to double strand breaks and are deficient in repairing this type of DNA damage (Zhong et al., 2002). BRCA1 represses E2 responsive gene expression and ER transcriptional activity, which may link the functions of BRCA1 to specific target tissues (Fan et al., 1999). BRCA1 can bind directly to ER independently of E2 via the amino terminus of the tumor suppressor and the carboxyl domain of the receptor (Fan et al., 2001). Amino terminal truncations of BRCA1 blocked the ability of the tumor suppressor to inhibit ER activity in these studies. However our results using a mutant BRCA1 protein showed that despite an intact amino terminus, the truncated tumor suppressor was not able to inhibit E2 mediated increases in double strand break repair and cell survival. These data suggest a role for the BRCA1

carboxyl terminus in mediating the E2 dependent effects. We showed that this ligand mediated protection correlated with formation of ER/coactivator complexes with BRCA1. However RA treatment did not recruit BRCA1 to RAR/CBP heterodimers, suggesting a receptor specific effect. Our studies demonstrated that in the absence of the BRCT carboxyl domain, the mutant BRCA1 repressed expression of multiple double strand break repair proteins. The mechanisms by which these transcriptional complexes regulate DNA repair genes will be important in future studies.

Our results demonstrate that expression of a mutant BRCA1 construct inhibited cell cycle progression in human breast cancer cell lines which correlated with decreased sensitivity to double strand breaks. A previous study showed that loss of BRCA1 function in breast cancer resulted in cell cycle arrest through p53 and p21 (Sourvinos and Spandidos, 1998). In agreement with our results, several carboxyl terminal truncated BRCA1 proteins conferred chemoresistance and decreased susceptibility to apoptosis (Fan et al., 2001). However, a small carboxyl terminal BRCA1 truncation caused defective transcriptional activation, cell cycle progression, and increased sensitivity to double strand breaks in an ovarian cancer cell line (Zhou et al., 2003). These studies illustrate cell specific differences in BRCA1 function and that the carboxyl terminal domain should be better defined in order to understand its effects on these diverse cellular processes.

Our results demonstrated that E2 treatment resulted in complex formation between ERα, CBP, and BRCA1 in ER positive breast cancer cell lines. RA treatment recruited CBP but not BRCA1 to RARα in both ER positive and negative cell lines. The carboxyl terminal domain of CBP has been shown to interact in vitro and in vivo with BRCA1 (Cui et al., 1998). BRCA1 interaction with CBP and p300 was shown to occur in a phosphorylation independent manner through the CREB binding domain of the coactivators and both amino and carboxyl termini of the tumor suppressor (Pao et al., 2000). The ability of BRCA1 to repress ER responsive gene expression correlated with its ability to downregulate p300 but not CBP expression (Fan et al., 2002). Increased expression of CBP or p300 rescued the inhibition of ER responsive genes by BRCA1, perhaps by displacing BRCA1 from the nuclear receptor. Sequence comparisons between ER and RAR may reveal important differences between these receptors which functionally regulate their interactions with coactivators and BRCA1.

In the final year of the funded application, we will assess DNA damage levels in primary tumor cells from rat mammary cancers. The career development activities detailed in the application will also continue.

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